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(12) # EUROPEAN PATENT APPLICATION

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(54) **Cell culture method.**

(57) A cell culture method comprises cultivating cells, e.g. animal or plant cells or tissue, in a culture medium comprising microalgae, e.g. in the form of an extract of Chlorella, Scenedesmus or Spirulina.

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# CELL CULTURE METHOD

The present invention relates to a method of cell culture, e.g. of animal or plant cells or tissue.

The technique of so-called "successive cultivation" of animal tissue is known for many biological fields, including medicine and pharmacology, as an effective and practicable means of research. For this reason, the technique has been applied in research, e.g. in elucidating the mechanisms of, for example, cell differentiation and cancer.

For the successive cultivation of animal tissue, it is almost always necessary to use a serum of, for example, an adult, neonate or foetus of a subject which may be bovine or a horse, chicken or rabbit, in addition to a chemically-defined synthetic culture medium comprising, for example, aminoacids, vitamins and/or minerals. The need for a serum is based on the fact that it contains a cell multiplicative growth factor (a substance other than the general nutrient materials); therefore, multiplication of animal tissue cells is impossible without serum. Problems are associated with the use of serum, however; its instability and expense, for example.

When a piece of tissue cut out of a plant body exhibiting relatively rapid growth, such as undifferentiated shoot apex or root cambium, is cultivated in an artificial culture medium, callus formation, which is a phenomenon of undifferentiated cell multiplication, and the formation of differentiated foliate parts and root, are observed separately or simultaneously. In such a plant tissue culture, it is often attempted to enhance growth by the addition of a suitable substance such as coconut milk or yeast extract. The enhancement of growth is limited by the proliferation of the callus, and it has therefore been impossible to produce a differentiated plant body without empl ying any additional measure. In rder to facilitate differentiation, means such as, f r example, ultraviolet

irradiation and the addition of phytohormones such as indoleacetic acid or benzayladenine have been used. Such means are, however, difficult to control; in particular, synthetic phytohormones may either promote or retard differentiation, depending upon the amount which is used.

According to the present invention, a cell culture method comprises cultivating cells in a culture medium comprising microalgae.

As used in this specification, the term "microalgae" means unicellular algae or their near relatives such as, for example, Chlorella, Scenedesmus or Spirulina. The algae may be naturally-occurring or cultivated.

The microalgae are suitably introduced into the culture medium in the form of an extract which has been obtained, for example, by the solvent extraction of cells of one or more of types of microalgae.
The solvent is preferably aqueous, e.g. water itself or an aqueous solution of an acid, base or organic solvent.

In order to effect the extraction, algae cells may be brought into contact with the solvent which may be at ambient or elevated temperature. Hot water extraction is preferred. By way of example, algae cells are suspended in water in an amount of 1 to 1000 grams algae (dry weight) per liter of water and are maintained at 50 to 150°C for 0.5 to 120 minutes, preferably at about 100°C for more than a minute, and are then separated from the water by, for example, centrifugation. The resultant extract may be purified, if desired, by any suitable technique such as gel filtration or dialysis.

The extract usually contains sugars, proteins, polysaccharides and nucleic acids at least, various components having molecular weights in the range of from 1,000 to 1,000,000. Such extracts can exhibit multiplication pr moting activity in cultivating animal and plant, and the differentiation of plant, cells. The term "cells" will be

understood herein to include tissue.

The extract can be used per se, as a fraction obtained by molecular weight fractionation thereof, or in the form of a concentrate or dry powder obtained by, for example, freeze-drying or spray-drying. The preferred forms for use in the invention are powders, a high molecular fraction containing suguars, proteins and polysaccharides (especially for animal cells) and a high molecular fraction containing nucleic acids, glycoproteins and polysaccharides (especially for plant cells).

The culture process of the invention may follow the procedures of, and use the same basic culture media as are used in, conventional tissue culture. Thus, a known synthetic culture medium may be used.

For animal cells, the medium preferably contains aminoacids and vitamins at least in addition to the microalgae, and the cultivation is conducted under aseptic conditions. The culture medium may also contain serum, e.g. animal serum, but the amount which is needed may be no more than 10% of that previously considered to be necessary in cultivating animal cells; for example, a culture medium used in the present invention may contain no more than 1% of serum.

For plant cells, the culture medium preferably contains mineral nutrients and a carbon source at least in addition to the microalgae. The medium may also contain, for example, inorganic salts, micro or mineral nutrients, and vitamins. Further, growth-promoting agents such as coconut milk, and differentiation agents such as synthetic phytohormones, may also be included in the medium.

The amount of the microalgae present in the medium is often at least 0.3, and usually no more than 500, mg (dry weight) per liter of the medium. For animal cells, the preferred amount is from 0.3 to 400, and more preferably fr m 1 to 100, mg/l. For plant cells, the

preferred amount is from 1 to 500 mg/l.

Animal cells or tissue which may be cultured by the method of the invention include somatic cells from an animal body, e.g. normal or cancer cells. Plant cells or tissue to be cultured may be from any suitable plant, shoot apex, cambium and hypocotyl of seedlings being preferred. It is also possible to cultivate multiplicated but undifferentiated cells developed in plants, such as callus, and cells or tissue obtained by successive cultivation.

When animal cells are cultivated by the method of the invention, successive cultivation can be maintained by the multiplication promotion effect. The reason for this effect is not clear, but it is theorised that cell metabolism is stimulated by the action of some substance, probably of high molecular weight, such as a glycoprotein or polysaccharide, in the microalgae. This effect has the important advantage that the amount of any serum which is used need be very much less than has previously been the case, thereby reducing costs and facilitating handling. When plant cells such as shoot apex tissue are cultivated by the method of the invention, not only is callus proliferation enhanced, but also differentiation is promoted, so that plant body, stem, leaf or root may be developed from the callus even without callus formation. It is possible to harvest the multiplicated mass of cells and the grown body or other parts, or to transplant the differentiated plant body as a seedling. It may also be possible to extract useful materials from callus which may be obtained. Stem or leaf which may have been differentiated exclusively may have important applications. Well replicated/homogeneous seedling cultures may be obtained, allowing conservation of a plant species, for example.

The following Examples illustrate the invention.

Example 1

30 g of Chlorella powder were suspended in 1 l of water and subjected to hot water extraction at 100°C for 30 minutes. The suspension was then centrifuged and the supernatant (corresponding to 4.5 g dry extracted matter) was subjected to molecular fractionation on a Sephadex G-25 column. The fractions with molecular weights above 3,000 were adsorbed on a DEAE-cellulose column, and then eluted with 0.01 M carbonate buffer. A fraction having a component with a molecular weight over 70,000, a component with a molecular weight in the range from 30,000 to 10,000 which comprises rich neutral sugar, and a component with a molecular weight in the range 10,000 to 3,000 which comprises neutral sugar protein in nearly equivalent proportions, was obtained (hereinafter termed Fraction $A_1$). Fraction $A_1$ was freeze-dried to give 0.63 g ry matter. The molecular fractionation chart of fraction $A_1$ is given in Figure 1 of the accompanying drawings.

$5 \times 10^4$ of cell strain RLC-10 (rat liver cells) were cultivated in a culture medium prepared by adding a cattle foetus serum (FCS) in an amount of 10% to a sector (Sector C) of a basic culture medium, DM-160, whose composition is given in Table 1.

Table 1

Composition of Basic Culture Medium DM-160

| Amino Acids | mg/l | Vitamins | mg/l |
|---|---|---|---|
| Ala | 400 | $B_1$ | 1.0 |
| Arg | 100 | $B_2$ | 1.0 |
| Asp | 25 | $B_6$ | 1.0 |
| Asn | 25 | $B_{12}$ | 0.005 |
| Cys·HCl | 80 | Pantothenic acid | 1.0 |
| Glu | 150 | Nicotinamide | 1.0 |
| Gln | 300 | Biotin | 0.1 |
| Gly | 15 | Choline HCl | 5.0 |
| His | 30 | C | 1.0 |
| Ile | 150 | Folic acid | 1.0 |
| Leu | 400 | Inositol | 5.0 |
| Lys | 100 | | |
| Met | 80 | | |
| Phe | 80 | | |
| Pro | 12 | | |
| Ser | 80 | | |
| Thr | 100 | | |
| Trp | 40 | | |
| Tyr | 50 | | |
| Val | 85 | | |

In a similar manner, RLC-10 was cultivated in DM-160 (Sector T), to which had b en added 1% FCS and 5 µg/ml Fraction $A_1$. Cell multiplication was observed by comparison of the two cultivation sectors. The sectors showed a similar trend of multiplication of cells. The results obtained are shown graphically in Fig. 2.

Example 2

In a similar manner as in Example 1, cell strain JTC-15 (rat ascites hepatoma), JTC-2 (rat ascites hepatoma) RLG-1 (rat lung cells) and RLC-18 (rat liver cells) were examined for their multiplication rates in the medium employed in Example 1 with variable contents, i.e. 0.5 µg/ml, 5 µg/ml and 50 µg/ml, of Fraction $A_1$. The tendencies of the multiplication of the cells were the same. The results obtained are shown graphically in Fig. 3.

Example 3

Shoot apexes (one of the most rapidly growing tissues in a plant body, including a growing point) of carnation were excised and were each placed, in test tubes, on a culture medium. Cultivation was conducted under conditions comprising a 9 hour illumination period (2,000 lx) and a 15 hour dark period per day, at 25°C. The culture medium was the basal culture medium of Murashige & Skoog, the composition of which is given in Table 2, excluding $FeSO_4$ but to which had been added 2.0% sucrose, 0.8% agar-agar and various amounts of a Chlorella extract.

Table 2

Basal Culture Medium according to Murashige & Skoog

| Inorganic Salts (mg/l) | | Inorganic Salts (mg/l) | | Vitamins etc. (mg/l) | |
|---|---|---|---|---|---|
| $NH_4NO_3$ | 1650 | $H_3BO_3$ | 6.2 | Nicotinic acid | 0.5 |
| $KNO_3$ | 1900 | $MnSO_4.4H_2O$ | 22.3 | Pyridoxine-HCl | 0.5 |
| $CaCl_2.2H_2O$ | 440 | $ZnSO_4.4H_2O$ | 8.6 | Thiamine.HCl | 0.1 |
| $MgSO_3.7H_2O$ | 370 | KI | 0.83 | Myoinositol | 100 |
| $KH_2PO_4$ | 170 | $Na_2MoO_4.2H_2O$ | 0.25 | Glycine | 2 |
| $Na_2$-EDTA | 37.3 | $CuSO_4.5H_2O$ | 0.025 | | |
| $FeSO_4.7H_2O$ | 27.8 | $CoCl_2.6H_2O$ | 0.025 | | |

Each culture medium had a pH of 5.5 - 6.0. The Chlorella estract was obtained by dispersing 30 g of Chlorella dry powder in 1 l of water and subjecting the dispersion to hot water extraction at 100°C for 30 minutes, centrifuging the resulting suspension and freeze-drying the supernatant obtained (4.5 g dry weight). The ultraviolet absorption spectrum of the aqueous solution of this Chlorella extract is given in Fig. 4. The amounts of the extract added to the culture medium were respectively 0, 2, 6 and 20 mg/l.

After cultivation for 2 months, the greatest degree of multiplication and differentiation had occurred in the tissues containing the highest content of the extract.

When the same procedure was repeated using 100

test tube samples for each of the cases in which 0 and 20 mg/l of the extract were added to the medium, the cultured samples were classified as to whether (A) the whole tissue had become callus, without differentiation; (B) stem and leaf were formed with callused stem; (C) stem, leaf and root were formed with callused stem; (D) stem, leaf and root were formed without callus formation; and (E) neither multiplication nor differentiation was observed. Table 3 gives the results of the evaluation for these cultured samples.

Table 3

| Added Extract (mg/l) | Evaluation | | | | |
|---|---|---|---|---|---|
| | (A) | (B) | (C) | (D) | (E) |
| 0 | 22 | 22 | 11 | 34 | 11 |
| 20 | 15 | 31 | 46 | 8 | 0 |

From these results, a multiplication-promoting effect of the Chlorella extract is observed since the number of (D) and (E) evaluated samples is reduced by the addition of the extract. The relative (A), (B) and (C) figures indicate a differentiation-promoting effect.

Example 4

When naked rice seed is cultivated on an agar-agar culture medium containing 2.0 mg/l of the synthetic growth hormone 2,4-D, callus formation is observed. In this Example, the influence of the addition of microalgae extract upon such callus formation is examined.

Using a basal culture medium B-5 as identified in Table 4, culture media (pH 6.0) were prepared by adding thereto 2.0% by weight of sucrose, 2.0 mg/l of 2,4-D, 0.8% by weight of agar-agar, and the Chlorella extract in amounts

varying from 0 to 500 mg/l. The cultivation conditions were the same as in Example 1.

Table 4

Basal Culture Medium B-5 (pH 6.0)

| Component | Content | | Component | Content | |
|---|---|---|---|---|---|
| $NaH_2PO_4.2H_2O$ | 171.6 | mg/l | $Na_2MoO_4.2H_2O$ | 0.2522 | mg/l |
| $KNO_3$ | 2527.8 | mg/l | $CoCl_2.6H_2O$ | 0.0242 | mg/l |
| $(NH_4)_2SO_4$ | 132.1 | mg/l | KI | 0.7457 | mg/l |
| $MgSO_4.7H_2O$ | 246.5 | mg/l | Nicotinic Acid | 1.0 | mg/l |
| $CaCl_2.2H_2O$ | 111.0 | mg/l | Thiamine.HCl | 10.0 | mg/l |
| Fe-EDTA | 28 | mg/l | Pyridoxine.HCl | 1.0 | mg/l |
| $MnSO_4.H_2O$ | 13.6 | mg/l | m-Inositol | 100.0 | mg/l |
| $ZnSO_4.7H_2O$ | 1.98 | mg/l | Sucrose | 20.0 | g/l |
| $H_3BO_3$ | 3.03 | mg/l | 2,4-D | 2.0 | mg/l |
| $CuSO_4$ | 0.0393 | mg/l | | | |

30 days after the seeds were placed on the culture media, evaluation was made in the same manner as in Example 3. Table 5 show the results.

Table 5

| Added Extract (mg/l) | Evaluations | | | | |
|---|---|---|---|---|---|
| | (A) | (B) | (C) | (D) | (E) |
| 0 | 86 | 2 | 3 | 0 | 9 |
| 10 | 54 | 32 | 14 | 0 | 0 |

These results show that the extract promotes differentiation, since the number of samples in classes (B) and (C) are greater in that case, This observation was confirmed, and that a marked effect could be achieved, when

the extract was added to the medium in an amount ranging from 1 to 100 mg/l. It was also noted that the number of samples xhibiting cell multiplication was substantially independent of the presence of the extract, although the increase in the weight of the grown body was greater for those samples containing the extract than for those without; this shows that the extract promotes proliferation.

CLAIMS

1. A cell culture method, which comprising cultivating cells in a culture medium, characterised in the medium comprises microalgae.

2. A method according to claim 1, in which the microalgae are selected from Chlorella, Scenedesmus and Spirulina.

3. A method according to claim 1 or claim 2 in which the microalgae are introduced in the form of an aqueous extract.

4. A method according to any preceding claim, in which the cells are animal cells.

5. A method according to claim 4, in which the cells are selected from normal tissue and cancer cells.

6. A method according to claim 4 or claim 5, in which each litre of the medium comprises from 0.3 to 400 mg of the microalgae.

7. A method according to any of claims 4 to 6, in which the medium additionally comprises animal serum.

8. A method according to any of claims 1 to 3, in which the cells are plant cells.

9. A method according to claim 8, in which the cells have been subjected to successive cultivation.

10. A method according to claim 8 or claim 9, in which each litre of the medium comprises from 1 to 500 mg of the microalgae.

Gill Jennings & Every

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25th January 1982

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The Netherlands

Dear Sirs,

European Patent Application No. 81304612.5
Applicants: CHLORELLA INDUSTRY CO., LTD

We refer to the official letter of 16th December 1981 and file herewith a revised Declaration of Inventorship, giving Mr Tanaka's correct address. A copy of the Declaration, for forwarding to Mr Tanaka, is also enclosed.

We request an opportunity to correct various errors occuring in the specification, i.e. :

Page 2, line 2:
change "benzayladenine" to "benzyladenine";

Page 3, line 27:
change "micro or mineral nutrients," to "microelements";

Page 5, line 16:
change "ry"to "dry"; and

Page 9, line 29:
delete "2.0% by weight of sucrose, 2.0 mg/l of 2,4-D," (since these components are already specified in Table 4).

Alternatively, we request that the examiner makes the above amendments on our behalf.

Please date-stamp and return the enclosed copy of this letter to us, as proof of receipt.

Yours faithfully,
Gill Jennings & Every

R.E. Perry
Authorised Representative.

FIG.1

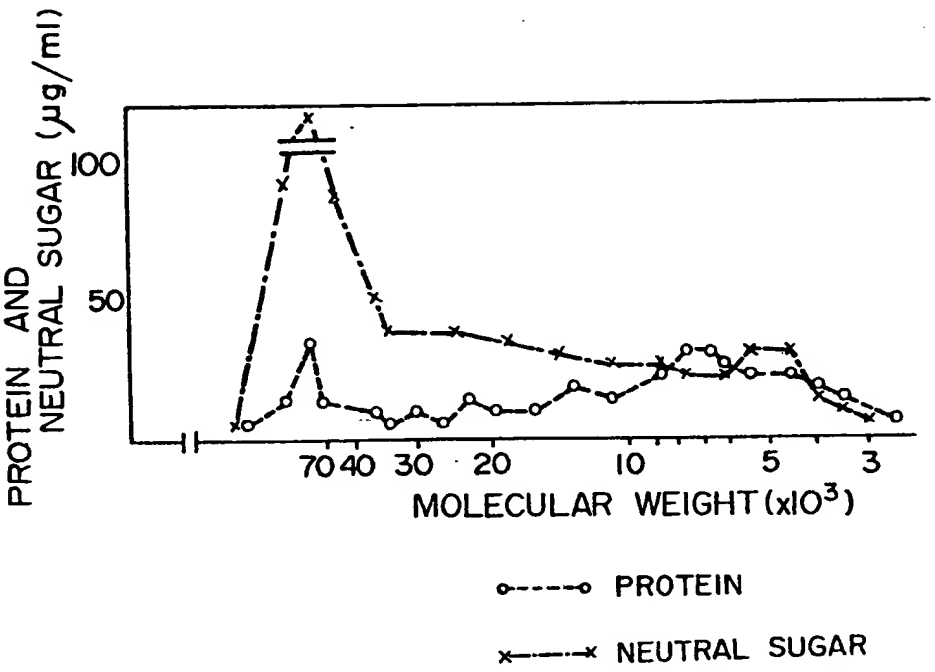
PROTEIN AND NEUTRAL SUGAR (μg/ml)
100
50
70 40 30 20 10 5 3
MOLECULAR WEIGHT (x10³)
PROTEIN
NEUTRAL SUGAR

FIG.2

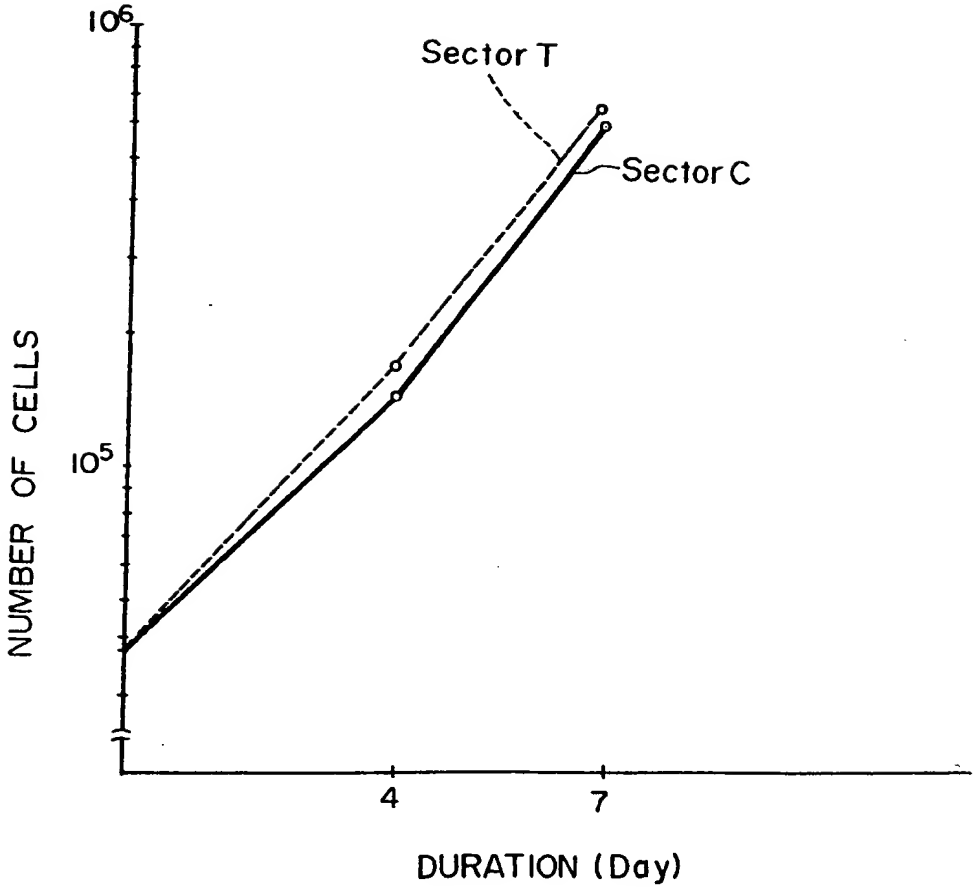

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FIG.3

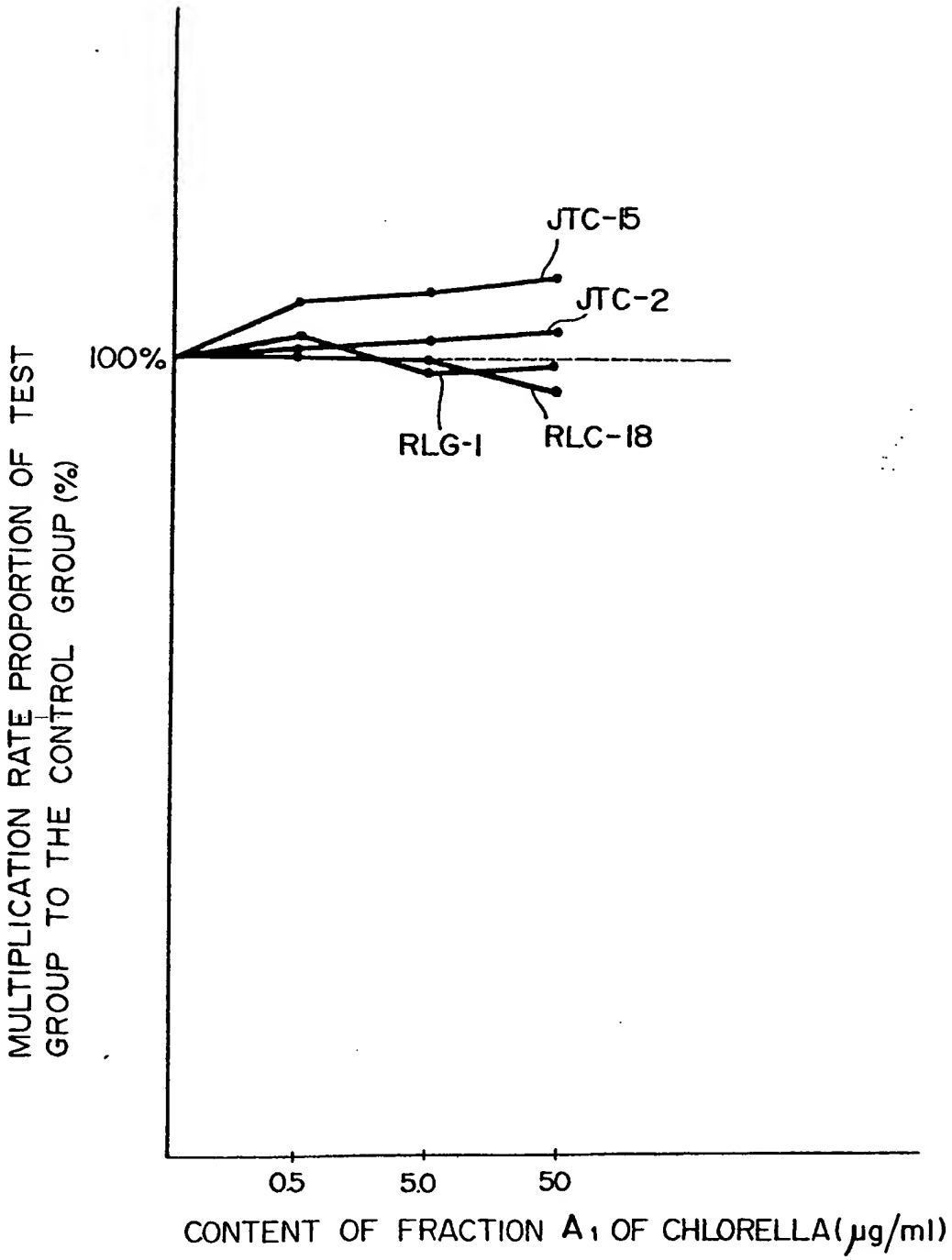
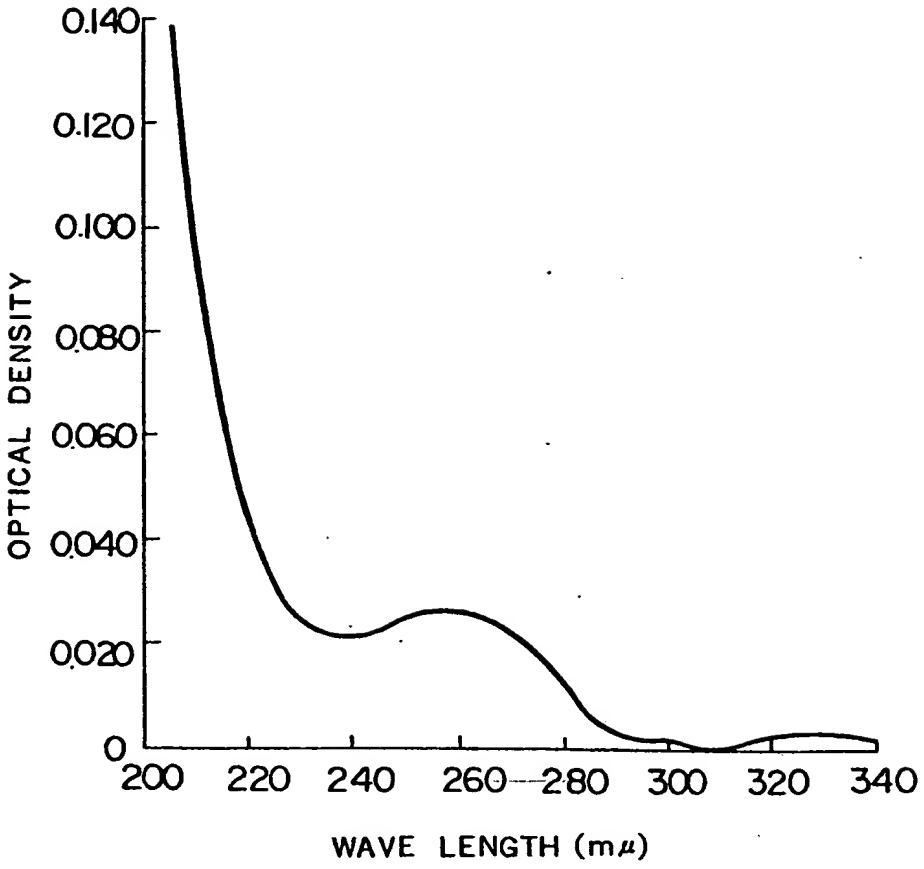


FIG. 4


0.140
0.120
0.100
0.080
0.060
0.040
0.020
0
OPTICAL DENSITY
200
220
240
260
280
300
320
340
WAVE LENGTH (mμ)